Confocal and Electron Microscopic Studies of Laser Subepithelial Keratomileusis (LASEK) in the White Leghorn Chick Eye

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Objectives: To evaluate the effect of 20% alcohol on the white leghorn chick cornea and to determine the confocal and electron microscopic findings of laser subepithelial keratomileusis surgery in the white leghorn chick corneal model.

Method: Laser subepithelial keratomileusis surgery was performed on chick corneas and the morphologic changes were examined by transmission electron microscopy. Chick corneas were exposed to 20% alcohol for 30 and 45 seconds or 1 and 2 minutes (5 chicks per group) to evaluate the effect on the corneal epithelium. Photorefractive keratectomy using either mechanical or 20% alcohol-assisted debridement (5 chicks per group) was also performed. Keratocyte and epithelial cell deaths were analyzed 4 hours after surgery using terminal deoxynucleotidyl transfer-mediated biotin-deoxyuridine 5-triphosphate nick-end labeling (TUNEL) staining and transmission electron microscopy.

Results: Exposure of the corneal epithelium to 20% alcohol for 30 seconds or longer allowed reproducible separation of epithelial flaps in white leghorn chick eyes. Transmission electron microscopy immediately after alcohol treatment showed that exposure to 20% alcohol for 30 seconds or less had minimal adverse effects on the corneal epithelium. The TUNEL staining of corneas obtained 4 hours after surgery revealed TUNEL-positive cells in the central superficial stroma and more abundantly in the peripheral superficial stroma around the epithelial flap margin and in the epithelial flap itself, particularly in the basal epithelial layer. Transmission electron microscopy showed similar evidence of apoptosis in the epithelium and anterior stroma.

Conclusions: The white leghorn chick eye seems to be a reasonable model for laser subepithelial keratomileusis surgery. Treatment with 20% alcohol for 30 seconds results in reproducible epithelial flap creation in the chick cornea and in relatively low levels of stromal and epithelial cell death after surgery.

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The excimer laser has provided safe and effective approaches for the correction of refractive errors. Photorefractive keratectomy (PRK) and laser in situ keratomileusis have been the most frequently performed surgical procedures for the treatment of refractive errors. Recently, laser subepithelial keratomileusis (LASEK) has been introduced; in this technique 18% to 20% alcohol is used to create an epithelial flap, followed by excimer laser ablation and repositioning of the flap. Potential advantages of LASEK over PRK are reduced pain and corneal subepithelial haze.

One obstacle to increase our understanding of the mechanism of action of LASEK and of evaluating the toxic effects of dilute alcohol has been the absence of an animal model. After failed attempts to create reproducible epithelial flaps in several animal models using the same methods as for human LASEK surgery, we have developed an animal model for LASEK in white leghorn chick eyes (which have Bowman layers similar to those in humans). In the present study we evaluated the effects of 20% alcohol on the epithelium and basement membrane zone after various exposure times. We also evaluated the effects of 20% alcohol during LASEK using confocal and electron microscopy in the white leghorn chick eye model; PRK with either mechanical or 20% alcohol-assisted debridement served as controls.

METHODS

Newborn white leghorn chicks, purchased from Truslow Farms (Chestertown, Md), received Pu-
rinal Lab Mill Start&Grow food and water ad libitum. The chicks were anesthetized with an intramuscular mixture of ketamine hydrochloride (20 mg/kg) and xylazine hydrochloride (5 mg/kg). For testing the effects of alcohol, chick corneas (20 eyes) were treated using 20% alcohol for 30 and 45 seconds or 1 and 2 minutes (5 chicks per time point). Ethanol was applied in 3-mm trephines. Chick eyes (20) also were examined in the following categories: untreated, PRK with epithelial scrape using mechanical debridement, 20% alcohol-assisted debridement, and LASEK (5 chick eyes per category). All experiments were conducted according to the Association of Vision Research in Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and on a protocol approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary, Boston.

The chick eyes were propsected anteriorly to the eyelids after medial and lateral canthotomy. For PRK with mechanical and 20% alcohol-assisted epithelial debridement, a 3-mm trephine was used to demarcate the central cornea, which was followed by epithelial debridement using a No. 15 Bard-Parker blade (Becton Dickinson, Franklin Lakes, NJ).

For PRK with 20% alcohol-assisted epithelial debridement or the creation of an epithelial flap for LASEK, the 3-mm trephine was applied on the cornea, and several drops of 20% alcohol were instilled, enough to cover the entire corneal epithelium. After 30 seconds, the trephine was removed and the eye was irrigated with a balanced salt solution. One arm of a jeweler’s forceps was then inserted under the epithelium and traced around the delineated margin of the epithelium, leaving 2- to 3-clock hours of intact margin. The loosened epithelium was peeled as a single sheet using a dry sponge (Meroxel; Xomed, Jacksonville, Fl) and jeweler’s forceps, leaving a flap of epithelium with the hinge still attached. After laser ablation, a 30-gauge anterior chamber cannula (Becton Dickinson) was used to hydrate the stroma and epithelial flap with a balanced salt solution. The epithelial flap was replaced on the stroma and allowed to dry for 1 minute. Then the lower eyelid was pulled and repositioned to prevent excess dehydration from the exposure. For the excimer laser ablation on the stroma, a 2-mm-diameter, 4-µm-deep (approximately 5% of the thickness of the chick cornea) ablation was performed with an excimer laser (Apex SVS; Summit, Waltham, Mass) in both PRK and LASEK procedures. The pulse energy density was 160 mJ/cm² and the repetition rate was 10 Hz. The excimer laser was programmed with a phototherapeutic keratectomy. The chicks were killed with an excess dose of phenobarbital sodium immediately after the end of each procedure. The corneoscleral rim of each eye was removed with surgical scissors for transmission electron microscopy (TEM) and embedded in OCT compound (Sakura Finetek, Torrance, Calif) 4 hours after surgery for terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine 5-triphosphate nick-end labeling (TUNEL) assay.

TRANSMISSION ELECTRON MICROSCOPY

For TEM, the corneoscleral rims were fixed in half-strength Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde) and processed in 0.2M cacodylate buffer (pH 7.4) overnight. The samples were then postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, embedded in epoxy resin (Epon 812; Epon-LKB Instrument, Gaithersburg, MD; and Araldite 506; Ernest F. Fullam, Latham, NY) and oven-dried at 60°C for 48 hours. Sections 1-µm thick were stained with toluidine blue for orientation. Subsequent ultrathin sections were obtained using an ultramicrotome counterstained with 2% uranyl acetate–lead citrate, and analyzed using a TEM (model 410; Philips, Eindhoven, the Netherlands).

The effect of 20% alcohol on the basement membrane zone is shown in Figure 1. Untreated white leghorn chick corneas had intact Bowman layer and the basal lamina was unilamellar. After 45 seconds exposure to 20% alcohol, there was evidence of disruptions of the lamina densa. After 2 minutes’ exposure, the basement membrane layer showed discontinuities and dense granules in the basal lamina (Figure 1).

Deleterious effects of 20% alcohol on the superficial epithelial cell layers were noted after exposure for 45 seconds. After 2 minutes’ exposure, widespread epithelial cell damage and disappearance of microvilli were evident (Figure 2).

Figure 3 shows the effect of 20% alcohol on the epithelial flap and underlying stroma immediately after creation of a LASEK flap. Figure 4 and Figure 5 show the effect of 20% alcohol on the epithelial flap and underlying stroma 4 hours after LASEK and PRK. Terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine 5-triphosphate–digoxigenin nick-end labeling assay showed evidence of TUNEL-positive cells in the basement membrane region after LASEK suggesting positive DNA fragmentation consistent with apoptosis. No keratocyte or epithelial cell staining was seen in untreated cornea (Figure 4). Four hours after PRK with mechanical or 20% alcohol-assisted epithelial debridement, superficial keratocytes showed evidence of TUNEL positivity. The TUNEL-positive keratocytes were observed in the central superficial anterior stroma after LASEK, and more abundant TUNEL-positive keratocytes were seen in the peripheral superficial stroma around the epithelial flap margin (Figure 4).

RESULTS

Eight-micrometer-thick sections were placed on microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, Pa). An in situ cell death detection kit for TUNEL assay was purchased from Roche Diagnostics (Indianapolis, Ind). The TUNEL staining, which detects cell death by apoptosis predominantly and by necrosis to a lesser extent, used an in situ fluorescein-based apoptosis detection kit and followed procedures recommended by the manufacturer. A laser scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany) was used to photograph all propidium iodide–stained cell nuclei (as red fluorescence) for nuclear labeling and the fluorescein-stained cell nuclei (as green fluorescence) for apoptosis.

In the past 5 years, PRK and laser in situ keratomileusis (LASIK) have become the most commonly performed refractive surgical procedures.3,4,7 Photorefractive keratectomy is a relatively safe procedure; its major limitations are postoperative pain, subepithelial haze, and prolonged visual rehabilitation.3,4,12,17 Laser in situ keratomileusis offers less discomfort, faster visual rehabilitation, and minimal haze, but it has its own set of complications, predominantly related to the flap.16,21 Laser subepithelial keratomileusis is an alternative to these 2 refractive surgical procedures; it avoids the flap complications of LASIK.

COMMENT
and reduces the pain and corneal haze of conventional PRK.8-11,22 Laser subepithelial keratomileusis is particularly suitable in patients with narrow palpebral fissure, thin corneas, and professions or lifestyles, such as contact sports athletes and military personnel, that predispose to flap trauma.8,9 Patients with low myopia who are at a lower risk for subepithelial haze may also benefit from LASEK.

We have attempted to answer one of these questions: we developed an animal model for LASEK using the white leghorn chick to determine epithelial toxic effects and cell survival after alcohol treatment. We were able to make an epithelial flap using 20% alcohol for longer than 30 seconds. Unlike the rat, mouse, and rabbit, chick corneas have a Bowman membrane.23 Whether the Bowman layer may be a visible indicator of ongoing stromal-epithelial interactions or may have a function is unclear, but it allows creation of an epithelial flap after exposure to 20% alcohol.

In LASEK for humans, the exposure time to 18% to 20% alcohol is 25 and 30 seconds.8,9 To evaluate the effect of 20% alcohol on the epithelium and mechanical manipulation during the creation of the epithelial flap in LASEK, we carried out electron microscopy studies and TUNEL staining on white leghorn chick eyes.

Gabler et al24 have reported that the corneal epithelial flap was vital up to 45 seconds’ exposure to 20% alcohol using 0.1% trypan blue staining in human cadaver eyes. We found that 20% alcohol for 30 seconds resulted in a slight effect in the superficial epithelium, basal epithelial layers, and basement membrane zone. Since preservation of epithelial integrity and adherence is important to protect the underlying ablated stroma, an exposure time of 30 seconds to 20% alcohol may be better than 45-seconds exposure times.24 Prolonged exposure to 20% alcohol might cleave anchoring filaments and lamina densa, which implies that the plane of separation is likely to be between epithelial layer and basement membrane. Gabler et al24 noted that the plane of action of alcohol in human eyes was between the lamina densa and the Bowman layer. Their findings suggested that the alcohol used during LASEK may cleave the anchoring fibrils between the Bowman layer and corneal epithelial basement membrane. Our data from humans showed that the separation plane is not constant and may occur between the epithelium and basement membrane or between the basement membrane and the Bowman layer.8,23 This may be in part due to the variability of the shearing forces on the epithelium during surgery. In the chick eye, our findings demonstrate that the predominant separation plane is between the basal epithelial layers and the basement membrane.

The adherence of the basement membrane to the basal layer of the epithelium is of significance because it is believed that the basement membrane provides the stability and support that keeps the epithelium intact even with

Figure 1. Transmission electron micrographs of the basement membrane zone and the Bowman layer of white leghorn chick corneas. A, Untreated; a unilamellar basement membrane is noted. The cell membrane of the basal epithelial cell shows minimal undulations and is lined by electron-dense hemidesmosomes (arrow). B, After 30 seconds’ exposure to 20% alcohol, minimal change in the basement membrane zone is noted. C, After 45 seconds’ exposure, disruptions of the lamina densa are noted (arrow). The region of the basal lamina and the epithelial basal epithelial cell layer appears less well defined than in untreated corneas. D, After 1-minute exposure to 20% alcohol. Note the space between the basal lamina and the basal epithelial cell layer (arrow). E, After 2 minutes’ exposure, the basement membrane layer is discontinuous. Irregular granular extracellular matrix fragments (arrow) are evident across the corneal basement membrane zone. Bars indicate 1 µm.
Figure 2. Transmission electron micrographs of the superficial epithelial cells and the Bowman layer of white leghorn chick corneas. A, Untreated chick cornea showed the typical 5 to 7 epithelial cell layers with microplicae on the superficial corneal epithelial cells and the acellular Bowman layer (arrow). B, Appearance of corneal epithelial cells after 30 seconds' exposure to 20% alcohol. Note minimal changes in the superficial epithelial cells. C, After 45 seconds' exposure, the top 2 to 3 layers of the epithelium are damaged. D, Similar appearance of the corneal epithelial cells after 1-minute exposure to 20% alcohol. E, Appearance of epithelial cells after 2 minutes' exposure showing widespread damage of corneal epithelial cells. Bars indicate 10 µm.
manipulation, thereby preserving the integrity and viability of the entire corneal epithelium.\(^8\) The epithelial attachment to the basement membrane is important for cell survival in other tissues.\(^{26,27}\) The presence of hemidesmosomes also provides a possible anchoring mechanism for the epithelium to adhere to the ablated stroma.

After mechanical debridement of the corneal epithelium, an underlying keratocyte loss occurs within an hour. This was first recognized by Dohlman et al,\(^{28}\) and subsequently, many groups have investigated this phenomenon and attempted to reverse its outcome.\(^{29-33}\) Wilson et al\(^{32}\) first provided evidence that removal of the corneal epithelium by mechanical scraping can induce keratocyte apoptosis. This finding explains why there is an early loss of keratocytes in the anterior corneal stroma after epithelial scraping. The dead keratocytes are replenished within a few days through proliferation and migration of the remaining keratocytes, but the new keratocytes are activated and produce more collagen and glycosaminoglycan,\(^{34-36}\) which can cause corneal haze. Keratocyte loss, after removal of the corneal epithelium, can be prevented by a collagen shield alone or in combination with topical application of a corneal preservation medium.\(^{37}\) Park and Tseng\(^{38}\) reported that amniotic membrane precludes polymorphonuclear leukocyte infiltration and decreases lipid peroxidation and keratocyte death. Mohan et al\(^{39}\) demonstrated that if mouse or rabbit corneas are enucleated and washed with a phosphate-buffered saline solution and the corneal epithelium is then scraped, the keratocytes undergo apoptosis. This contradicts the conclusions of earlier work by Zhao et al\(^{40}\) who suggested that tears may be important in the induction of keratocyte loss after deepithelialization in the mouse cornea. They also showed that directly beneath some epithelial islands, many keratocyte nuclei seem to have survived, even in the presence of tears. These results suggest that any kind of mechanical barrier, such as the epithelial flap in LASEK over the bare stroma, might reduce keratocyte death after epithelial debridement.

Our study in chick eyes demonstrates that the presence of an epithelial flap over the central stroma caused minimal superficial keratocyte death even after excimer laser irradiation. Electron microscopy and TUNEL staining findings suggest that the basal epithelial cell layer may be the most fragile layer of the epithelium and the most likely to be damaged during creation of the epithelial flap. One may speculate that the presence of epithelium over the bare stroma may confine the epithelium-stromal interaction to only around the epithelial flap margin. After epithelial injury, although some cytokines such as Fas ligand\(^{41}\) and interleukin 1\(^{12}\) are released, they react only with the peripheral epithelial flap margin, which would cause reduced keratocyte loss and apoptosis in the peripheral superficial stroma after surgery. In addition, if an epithelial flap is preserved, it may act as a mechanical barrier. One may also speculate that the epithelial flap reduces mechanical trauma to the epithelium and the corneal surface and may play a role in reducing scar formation as compared to PRK.\(^{32}\) That diminishes the corneolacrimal reflex and reduces the influx of tear fluid, which has many factors such as soluble Fas antigen and Fas ligand,\(^{32}\) transforming growth factor \(\beta\),\(^{43,44}\) and tumor necrosis factor \(\alpha\).\(^{45,46}\)

In addition to its barrier function, the epithelium may also be a source of active molecules that protect the keratocytes, or it may serve as a neutralization site for factors that would trigger keratocyte death. The viabil-

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**Figure 3.** Transmission electron micrographs of the epithelial flap and underlying stroma of the chick corneas immediately after creation of the flap using 20% alcohol showing the superficial epithelial cell layers (A) and the basement membrane zone (B). Bars indicate 10 µm.
ity of the corneal epithelial cells after alcohol exposure may also determine the extent to which the epithelial flap is beneficial after LASEK. Chen et al have demonstrated epithelial viability after LASEK in human epithelial sheets and in a monolayer of immortalized human corneal epithelial cells exposed to dilute alcohol. Signifi-
cant reductions of viable cells occurred when the duration of alcohol concentration exceeded 25% or when the duration of application of 20% alcohol exceeded 35 seconds. This was also associated with increased TUNEL positivity 8 to 24 hours after alcohol exposure. The current in vivo study using the white leghorn chick eye confirms the conclusions of Chen et al and allows better extrapolation of the experimental results to LASEK in humans.

Additional studies with longer follow-up periods may be necessary to determine the viability of the epithelium and the mechanism of its adhesion to the stroma after LASEK, which may help improve the safety, efficacy, and predictability of this surgical technique.

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